MYCOTAXON

ISSN (print) 0093-4666 (online) 2154-8889 Mycotaxon, Ltd. ©2018

January–March 2018—Volume 133, pp. 183–196

https://doi.org/10.5248/133.185

Chinese black truffles: *Tuber yigongense* sp. nov., taxonomic reassessment of *T. indicum* s.l., and re-examination of the *T. sinense* isotype

Li Fan[1*A], Jun-li Zhang[2], Ting Li[1],
Hui-juan Sun[2], Wei-ping Xiong[2*B], Yu Li[3]

[1] *College of Life Science, Capital Normal University,*
105 Xisanhuanbeilu, Haidian, Beijing 100048, China

[2] *Institute of Vegetable Sciences, Tibet Academy of Agricultural & Animal Husbandry Sciences,*
Lhasa, 850002, Tibet, China

[3] *Institute of Mycology, Jilin Agricultural University, Changchun 130118, China*

[*] Correspondence to: [A] *fanli@mail.cnu.edu.cn, lifan1966@hotmail.com*
[B] *13989009985@163.com*

Abstract—A new species, *Tuber yigongense,* is described. ITS sequences derived from Chinese black truffles grouped into three clusters, each recognised as a different species: *T. sinense*, *T. formosanum*, and *T. yigongense*. *Tuber sinense* and *T. formosanum* cannot be separated morphologically, but *T. yigongense* differs from them by its yellow brown ascospores. Based on ITS sequence analysis, numerous Chinese specimens previously identified as *T. indicum* and *T. himalayense* are redetermined as either *T. sinense* or *T. formosanum*; although the phylogenetic results suggest that the two Indian species do not occur in China, the true taxonomic affinities cannot be fully resolved because of the lack of Indian sequences of *T. indicum* and *T. himalayense*.

Key words—*Ascomycetes,* biogeography, DNA sequence, taxonomy, *Tuberaceae*

Introduction

Although Chinese truffles have been finding their way onto restaurant tables around the world over the past two decades, there is still confusion as to which taxonomic names should be applied. *Tuber indicum* Cooke & Massee was described from Mussooree [Mussoorie], near Dehradun in northwest India (Cooke & Massee 1892), and a second Indian species, based on a specimen from the same locality, was described as *Tuber himalayense* B.C. Zhang & Minter (Zhang & Minter 1988). In 1988 some specimens from Sichuan province were sent to Shanxi University and described as a new species *Tuber sinense* K. Tao & B. Liu based on morphological features (Tao & al. 1989). Prof. M. Zang identified a similar collection from Yunnan province as *T. indicum* (Zang 1992). Prof. Zang's opinion was widely accepted in China, and *T. sinense* was regarded as a synonym of the Indian species (Comandini & Pacioni 1997, Zhuang 1998, Riousset & al. 2001, Zhang & al. 2005, Song 2005, Chen 2007, Hall & al. 2007, Kinoshita & al. 2011, Bonito & al. 2013, Li & al. 2015, Zambonelli & al. 1997). Dr. H.T. Hu, who also found black truffles on Taiwan Island about the same time, proposed to name these "*Tuber formosanum*" H.T. Hu (Hu 1992), but this name was invalid as no type was assigned. It was later validly re-described as *T. formosanum* H.T. Hu & Y. Wang (Qiao & al. 2013). Because of its morphological similarity to the other Chinese black truffles, *T. formosanum* has also been treated as a synonym of *T. indicum* by some authors (Song 2005, Chen 2007, Cao 2010).

Molecular phylogenetic analyses have revealed that there are at least two distinct lineages in Chinese black truffles, strongly suggesting that there are two species. The two lineages have been interpreted in four different ways: (1) as "*Tuber indicum*-A" and "*Tuber indicum*-B" (Kinoshita & al. 2011, Bonito & al. 2013, Belfiori & al. 2013); (2) as two geographical ecotypes (Wang & al. 2006, Chen & al. 2016); (3) as two cryptic species (Chen & al. 2011, 2016); or (4) as *T. indicum* and *T. himalayense* (Zhang & al. 2005, Feng & al. 2016).

In this study, phylogenetic analyses were conducted on the Chinese black truffles using the ITS-nrDNA sequence, the popularly accepted DNA marker for species delimitation in *Tuber* (Bonito & al. 2010, 2013; Kinoshita & al. 2011; Fan & al. 2016a,b), in order to clarify the species composition of the Chinese black truffles and apply the correct scientific names. The analyses also revealed a new species and some morphological differences between *T. indicum* and Chinese taxa.

Materials & methods

Morphological studies

Fresh truffles were collected from under *Pinus* spp. or *Castanea* spp. in mixed woodlands in Hebei, Yunnan, Sichuan, and Tibet, China. Specimens were preserved in the Herbarium, Biology Department, Capital Normal University, Beijing, China

TABLE 1. Newly generated ITS gene sequences from specimens representing *Tuber* species sampled in the phylogenetic study.

TAXON	VOUCHER	ORIGIN	GENBANK NO.
T. formosanum	BJTC FAN107	Yunnan, China	MF621549
	BJTC FAN214	Yunnan, China	MF627984
	BJTC FAN329	Sichuan, China	MF627985
	BJTC FAN356	Sichuan, China	MF627986
	BJTC FAN363	Yunnan, China	MF627987
	BJTCFAN720	Hebei, China	MF627988
	BJTC FAN734	Hebei, China	MF627989
	BJTC FAN735	Hebei, China	MF627990
	BJTC FAN736	Hebei, China	MF627991
	BJTC FAN737	Hebei, China	MF627992
	BJTC FAN738	Hebei, China	MF627993
	BJTC FAN739	Hebei, China	MF627994
	BJTC FAN740	Hebei, China	MF627995
	BJTC FAN741	Hebei, China	MF627996
	BJTC FAN742	Hebei, China	MF627997
	BJTC FAN743	Hebei, China	MF627998
	BJTC FAN744	Hebei, China	MF627999
	BJTC FAN745	Hebei, China	MF628000
	BJTC FAN746	Hebei, China	MF628001
	BJTC FAN747	Hebei, China	MF628002
	BJTC FAN748	Hebei, China	MF628003
	BJTC FAN749	Hebei, China	MF628004
	BJTC FAN750	Hebei, China	MF628005
	BJTC FAN751	Hebei, China	MF628006
	BJTC FAN752	Hebei, China	MF628007
	BJTC FAN753	Hebei, China	MF628008
T. pseudohimalayaense	BJTC FAN122	Sichuan, China	MF627983
T. sinense	BJTC FAN108	Yunnan, China	MF627968
	BJTC FAN109	Yunnan, China	MF627969
	BJTC FAN110	Yunnan, China	MF627970
	BJTC FAN111	Yunnan, China	MF627971

Table 1 concluded on next page

Table 1, concluded

TAXON	VOUCHER	ORIGIN	GENBANK NO.
	BJTC FAN112	Yunnan, China	MF627972
	BJTC FAN114	Yunnan, China	MF627973
	BJTC FAN115	Sichuan, China	MF627974
	BJTC FAN116	Sichuan, China	MF627975
	BJTC FAN117	Yunnan, China	MF627976
	BJTC FAN118	Yunnan, China	MF627977
	BJTC FAN119	Yunnan, China	MF627978
	BJTC FAN159	Yunnan, China	MF627979
	BJTC FAN261	Yunnan, China	MF627980
	BJTC FAN488	Yunnan, China	MF627981
	HMAS 60222 [**IT**]	Sichuan, China	MF627982
T. yigongense	BJTC FAN728	Tibet, China	MF663717
	BJTC FAN729	Tibet, China	MF663716
	BJTC FAN730	Tibet, China	MF663715
	BJTC FAN731 [**T**]	Tibet, China	MF663714

[T] = holotype: [**IT**] = isotype

(BJTC). Fresh specimens were evaluated macroscopically, and microscopic characters were described from both fresh and rehydrated specimens. Microscopic characteristics were observed from 10–20 µm thick hand-cut sections of dried specimens soaked in 5% KOH (w/v), Melzer's reagent and cotton blue in lactic acid. For scanning electron microscopy (SEM), spores were scraped from dried gleba, placed onto double-sided tape mounted directly on SEM stubs, coated with a 8-nm thick platinum-palladium film using a HITACHI E-1010 ion-sputter coater, and examined and photographed with a HITACHI S-4800 scanning electron microscope.

DNA extraction, PCR amplification, and sequencing

Herbarium specimens were crushed by shaking for 30 s at 30 Hz, 2–4 times using a MIXER MILL MM 301 in a 1.5 ml tube together with one 3 mm diam. tungsten carbide ball, and total genomic DNA was extracted using CTAB. The internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) was amplified using primers ITS1f/ITS4 (White & al. 1990, Gardes & Bruns 1993). DNA was amplified in 50 µl reactions containing DNA template 2 µl primer (10 µM) 2 µl each, 2× MASTER MIX 25 µl as follows: an initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 50°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were purified, sequenced, and edited by Beijing Zhongkexilin Biotechnology Co. Validated sequences are stored in the NCBI database (http://www.ncbi.nlm.nih.gov/) under the accession numbers listed in TABLE 1. Additional ITS sequences (FIG. 1), including all available sequences from Chinese black truffles, were downloaded from the NCBI database.

Phylogenetic analysis

An ITS-nrDNA dataset was compiled to identify and investigate the phylogenetic placement of the black truffle species. Sequences alignments for each locus were performed in webPRANK (Löytynoya & Goldman 2005, using the -F setting) under default parameters and manually edited at misaligned sites. Poorly aligned sites for final sequence alignments were identified and ambiguous sites were excluded by Gblocks v. 0.91b (Castresana 2000; using default options except "Allowed Gap Positions" = half) with default parameters.

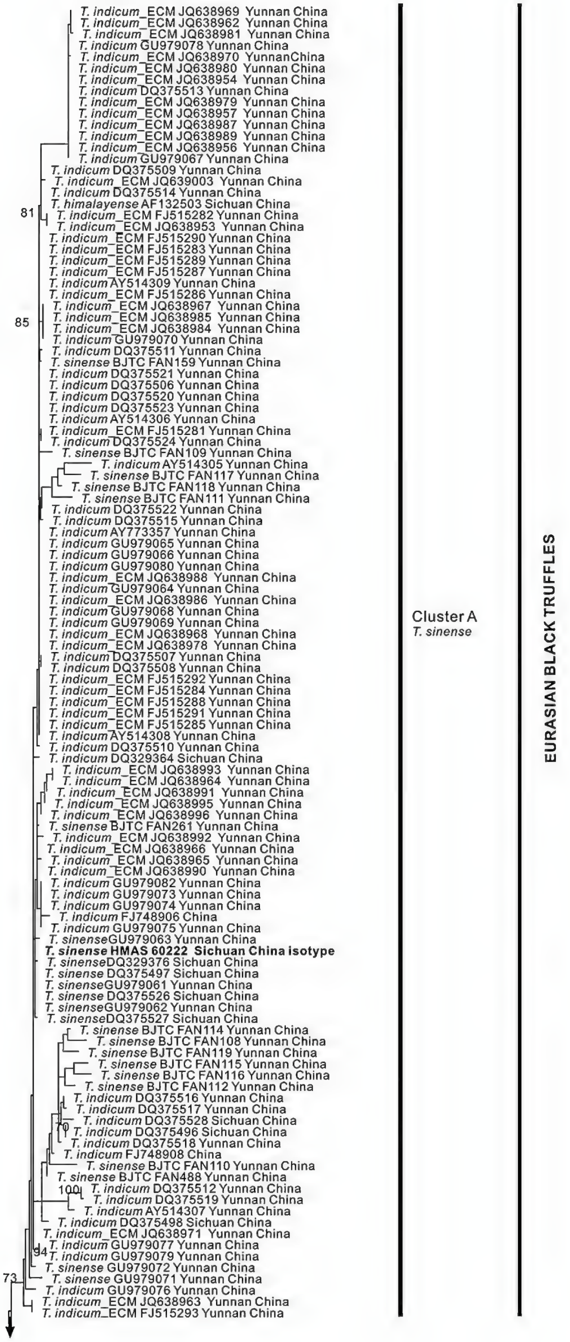
Maximum likelihood (ML) analysis was conducted using RAxML 8.0.14 (Stamatakis & al. 2005; Stamatakis 2006, 2014) and the GTRGAMMAI substitution model with parameters unlinked. ML bootstrap replicates (1000) were computed in RAxML using a rapid bootstrap analysis and search for the best-scoring ML tree. The ML trees were viewed with TreeView32 (Page 2001). Clades with a bootstrap support (BS) ≥70% were considered significantly supported (Hillis & Bull 1993).

Phylogeny

The ITS nrDNA alignment of 224 sequences comprised 575 characters; of the 370 variable sites, 60.18% (343) sites were informative. The resulting best ML tree (FIG. 1) grouped all sequences cited here (including two from European black truffle, others from Japanese black truffle, and all available GenBank ITS sequences derived from Chinese black truffles) into five clusters with strong support (BS ≥73%). The five clusters corresponded to European *T. melanosporum* Vittad., Japanese *Tuber* sp. 7 (Kinoshita & al. 2011), and three Chinese clusters designated as Clusters A–C (FIG. 1).

Cluster A contained our 15 newly generated *T. sinense* sequences (including the isotype sequence; TABLE 1) and 99 Chinese sequences [previously deposited as "*T. indicum*" (91), "*T. himalayense*" (1), or "*T. sinense*" (7)] (FIG. 1). Sequence similarity analysis showed that Cluster A sequences shared >98% ITS sequence identity and <94% similarity with other *Tuber* sequences.

Cluster B contained our 26 newly generated *T. formosanum* sequences (TABLE 1) and 55 Chinese sequences [previously deposited as "*T. indicum*" (49), "*T. himalayense*" (1), "*T. formosanum*" (3, including the holotype), or "*Tuber* sp." (2)] and two Japanese sequences [previously deposited as "*Tuber* sp. 6"] (FIG. 1). Cluster B sequences shared >98% ITS sequence identity and >94% similarity with other *Tuber* sequences.


T. indicum_ECM JQ638969 Yunnan China
T. indicum_ECM JQ638962 Yunnan China
T. indicum_ECM JQ638981 Yunnan China
T. indicum GU979078 Yunnan China
T. indicum_ECM JQ638970 YunnanChina
T. indicum_ECM JQ638980 Yunnan China
T. indicum_ECM JQ638954 Yunnan China
T. indicum DQ375513 Yunnan China
T. indicum_ECM JQ638979 Yunnan China
T. indicum_ECM JQ638957 Yunnan China
T. indicum_ECM JQ638987 Yunnan China
T. indicum_ECM JQ638989 Yunnan China
T. indicum_ECM JQ638956 Yunnan China
T. indicum GU979067 Yunnan China
T. indicum DQ375509 Yunnan China
T. indicum_ECM JQ639003 Yunnan China
T. indicum DQ375514 Yunnan China
T. himalayense AF132503 Sichuan China
81
T. indicum_ECM FJ515282 Yunnan China
T. indicum_ECM JQ638953 Yunnan China
T. indicum_ECM FJ515290 Yunnan China
T. indicum_ECM FJ515283 Yunnan China
T. indicum_ECM FJ515289 Yunnan China
T. indicum_ECM FJ515287 Yunnan China
T. indicum AY514309 Yunnan China
T. indicum_ECM FJ515286 Yunnan China
T. indicum_ECM JQ638967 Yunnan China
85
T. indicum_ECM JQ638985 Yunnan China
T. indicum_ECM JQ638984 Yunnan China
T. indicum GU979070 Yunnan China
T. indicum DQ375511 Yunnan China
T. sinense BJTC FAN159 Yunnan China
T. indicum DQ375521 Yunnan China
T. indicum DQ375506 Yunnan China
T. indicum DQ375520 Yunnan China
T. indicum DQ375523 Yunnan China
T. indicum AY514306 Yunnan China
T. indicum_ECM FJ515281 Yunnan China
T. indicum DQ375524 Yunnan China
T. sinense BJTC FAN109 Yunnan China
T. indicum AY514305 Yunnan China
T. sinense BJTC FAN117 Yunnan China
T. sinense BJTC FAN118 Yunnan China
T. sinense BJTC FAN111 Yunnan China
T. indicum DQ375522 Yunnan China
T. indicum DQ375515 Yunnan China
T. indicum AY773357 Yunnan China
T. indicum GU979065 Yunnan China
T. indicum GU979066 Yunnan China
T. indicum GU979080 Yunnan China
T. indicum_ECM JQ638988 Yunnan China
T. indicum GU979064 Yunnan China
T. indicum_ECM JQ638986 Yunnan China
T. indicum GU979068 Yunnan China
T. indicum GU979069 Yunnan China
T. indicum_ECM JQ638968 Yunnan China
T. indicum_ECM JQ638978 Yunnan China
T. indicum DQ375507 Yunnan China
T. indicum DQ375508 Yunnan China
T. indicum_ECM FJ515292 Yunnan China
T. indicum_ECM FJ515284 Yunnan China
T. indicum_ECM FJ515288 Yunnan China
T. indicum_ECM FJ515291 Yunnan China
T. indicum_ECM FJ515285 Yunnan China
T. indicum AY514308 Yunnan China
T. indicum DQ375510 Yunnan China
T. indicum DQ329364 Sichuan China
T. indicum_ECM JQ638993 Yunnan China
T. indicum_ECM JQ638964 Yunnan China
T. indicum_ECM JQ638991 Yunnan China
T. indicum_ECM JQ638995 Yunnan China
T. indicum_ECM JQ638996 Yunnan China
T. sinense BJTC FAN261 Yunnan China
T. indicum_ECM JQ638992 Yunnan China
T. indicum_ECM JQ638966 Yunnan China
T. indicum_ECM JQ638965 Yunnan China
T. indicum_ECM JQ638990 Yunnan China
T. indicum GU979082 Yunnan China
T. indicum GU979073 Yunnan China
T. indicum GU979074 Yunnan China
T. indicum FJ748906 China
T. indicum GU979075 Yunnan China
T. sinenseGU979063 Yunnan China
T. sinense HMAS 60222 Sichuan China isotype
T. sinenseDQ329376 Sichuan China
T. sinense DQ375497 Sichuan China
T. sinenseGU979061 Yunnan China
T. sinense DQ375526 Sichuan China
T. sinenseGU979062 Yunnan China
T. sinenseDQ375527 Sichuan China
T. sinense BJTC FAN114 Yunnan China
T. sinense BJTC FAN108 Yunnan China
T. sinense BJTC FAN119 Yunnan China
T. sinense BJTC FAN115 Yunnan China
T. sinense BJTC FAN116 Yunnan China
T. sinense BJTC FAN112 Yunnan China
T. indicum DQ375516 Yunnan China
T. indicum DQ375517 Yunnan China
T. indicum DQ375528 Sichuan China
70
T. indicum DQ375496 Sichuan China
T. indicum DQ375518 Yunnan China
T. indicum FJ748908 China
T. sinense BJTC FAN110 Yunnan China
T. sinense BJTC FAN488 Yunnan China
100
T. indicum DQ375512 Yunnan China
T. indicum DQ375519 Yunnan China
T. indicum AY514307 Yunnan China
T. indicum DQ375498 Sichuan China
T. indicum_ECM JQ638971 Yunnan China
T. indicum GU979077 Yunnan China
94
T. indicum GU979079 Yunnan China
T. sinense GU979072 Yunnan China
73
T. sinense GU979071 Yunnan China
T. indicum GU979076 Yunnan China
T. indicum_ECM JQ638963 Yunnan China
T. indicum_ECM FJ515293 Yunnan China
Cluster A
T. sinense
EURASIAN BLACK TRUFFLES

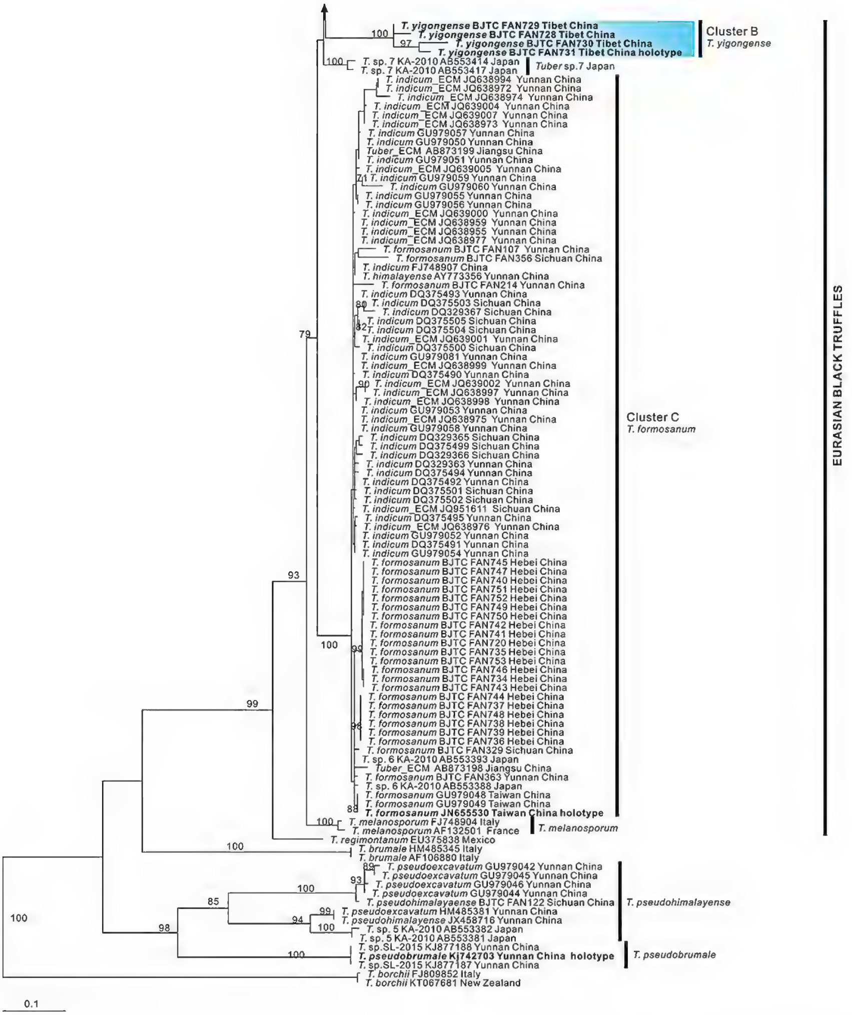

100
T. yigongense BJTC FAN729 Tibet China
T. yigongense BJTC FAN728 Tibet China
97
T. yigongense BJTC FAN730 Tibet China
T. yigongense BJTC FAN731 Tibet China holotype
Cluster B
T. yigongense
100
T. sp. 7 KA-2010 AB553414 Japan
T. sp. 7 KA-2010 AB553417 Japan
Tuber sp.7 Japan
T. indicum_ECM JQ638994 Yunnan China
T. indicum_ECM JQ638972 Yunnan China
T. indicum_ECM JQ638974 Yunnan China
T. indicum_ECM JQ639004 Yunnan China
T. indicum_ECM JQ639007 Yunnan China
T. indicum_ECM JQ638973 Yunnan China
T. indicum GU979057 Yunnan China
T. indicum GU979050 Yunnan China
Tuber_ECM AB873199 Jiangsu China
T. indicum GU979051 Yunnan China
T. indicum_ECM JQ639005 Yunnan China
71
T. indicum GU979059 Yunnan China
T. indicum GU979060 Yunnan China
T. indicum GU979055 Yunnan China
T. indicum GU979056 Yunnan China
T. indicum_ECM JQ639000 Yunnan China
T. indicum_ECM JQ638959 Yunnan China
T. indicum_ECM JQ638955 Yunnan China
T. indicum_ECM JQ638977 Yunnan China
T. formosanum BJTC FAN107 Yunnan China
T. formosanum BJTC FAN356 Sichuan China
T. indicum FJ748907 China
T. himalayense AY773356 Yunnan China
T. formosanum BJTC FAN214 Yunnan China
T. indicum DQ375493 Yunnan China
80
T. indicum DQ375503 Sichuan China
T. indicum DQ329367 Sichuan China
T. indicum DQ375505 Sichuan China
82
T. indicum DQ375504 Sichuan China
79
T. indicum_ECM JQ639001 Yunnan China
T. indicum DQ375500 Sichuan China
T. indicum GU979081 Yunnan China
T. indicum_ECM JQ638999 Yunnan China
T. indicum DQ375490 Yunnan China
90
T. indicum_ECM JQ639002 Yunnan China
T. indicum_ECM JQ638997 Yunnan China
T. indicum_ECM JQ638998 Yunnan China
T. indicum GU979053 Yunnan China
T. indicum_ECM JQ638975 Yunnan China
T. indicum GU979058 Yunnan China
Cluster C
T. formosanum
T. indicum DQ329365 Sichuan China
T. indicum DQ375499 Sichuan China
T. indicum DQ329366 Sichuan China
T. indicum DQ329363 Yunnan China
T. indicum DQ375494 Yunnan China
T. indicum DQ375492 Yunnan China
T. indicum DQ375501 Sichuan China
T. indicum DQ375502 Sichuan China
T. indicum_ECM JQ951611 Sichuan China
T. indicum DQ375495 Yunnan China
T. indicum_ECM JQ638976 Yunnan China
T. indicum GU979052 Yunnan China
T. indicum DQ375491 Yunnan China
T. indicum GU979054 Yunnan China
93
T. formosanum BJTC FAN745 Hebei China
T. formosanum BJTC FAN747 Hebei China
T. formosanum BJTC FAN740 Hebei China
T. formosanum BJTC FAN751 Hebei China
T. formosanum BJTC FAN752 Hebei China
T. formosanum BJTC FAN749 Hebei China
T. formosanum BJTC FAN750 Hebei China
T. formosanum BJTC FAN742 Hebei China
T. formosanum BJTC FAN741 Hebei China
100
T. formosanum BJTC FAN720 Hebei China
99
T. formosanum BJTC FAN735 Hebei China
T. formosanum BJTC FAN753 Hebei China
T. formosanum BJTC FAN746 Hebei China
T. formosanum BJTC FAN734 Hebei China
T. formosanum BJTC FAN743 Hebei China
99
T. formosanum BJTC FAN744 Hebei China
T. formosanum BJTC FAN737 Hebei China
T. formosanum BJTC FAN748 Hebei China
98
T. formosanum BJTC FAN738 Hebei China
T. formosanum BJTC FAN739 Hebei China
T. formosanum BJTC FAN736 Hebei China
T. formosanum BJTC FAN329 Sichuan China
T. sp. 6 KA-2010 AB553393 Japan
Tuber_ECM AB873198 Jiangsu China
T. formosanum BJTC FAN363 Yunnan China
T. sp. 6 KA-2010 AB553388 Japan
T. formosanum GU979048 Taiwan China
T. formosanum GU979049 Taiwan China
88
T. formosanum JN655530 Taiwan China holotype
100
T. melanosporum FJ748904 Italy
T. melanosporum AF132501 France
T. melanosporum
T. regimontanum EU375838 Mexico
100
T. brumale HM485345 Italy
T. brumale AF106880 Italy
EURASIAN BLACK TRUFFLES
89
T. pseudoexcavatum GU979042 Yunnan China
T. pseudoexcavatum GU979045 Yunnan China
93
T. pseudoexcavatum GU979046 Yunnan China
100
T. pseudoexcavatum GU979044 Yunnan China
85
T. pseudohimalayaense BJTC FAN122 Sichuan China
T. pseudohimalayense
99
T. pseudoexcavatum HM485381 Yunnan China
100
94
T. pseudohimalayense JX458716 Yunnan China
98
100
T. sp. 5 KA-2010 AB553382 Japan
T. sp. 5 KA-2010 AB553381 Japan
T. sp.SL-2015 KJ877188 Yunnan China
100
T. pseudobrumale Kj742703 Yunnan China holotype
T. pseudobrumale
T. sp.SL-2015 KJ877187 Yunnan China
T. borchii FJ809852 Italy
T. borchii KT067681 New Zealand
0.1

Fig. 1 (left+right). Phylogeny derived from maximum likelihood analysis of the ITS rDNA sequences of Eurasian black truffle species and related *Tuber* species, using *T. borchii* as outgroup. Bootstrap values >70% from 1000 replications are shown above the branches.

Cluster C, containing four sequences derived from our newly proposed *Tuber yigongense,* shared >99% ITS sequence identity and <93% similarity with other *Tuber* sequences.

Each of the three Clusters A–C represents a single species, according to the *Tuber* species delimitation criterion of 95% sequence identity suggested by Kinoshita & al. (2011).

Taxonomy

Tuber yigongense L. Fan & W.P. Xiong, **sp. nov.** FIG. 2a–d

FUNGAL NAME FN570492

Differs from other *Tuber* species by its dark brown to blackish ascomata and its yellow brown ascospores with a densely spiny reticulatum.

TYPE—China, Tibet, Linzhi City, Bomi County, Yigong Town, Jiangsegang Village, 2230 m, in soil under *Pinus* sp., 15 Nov. 2016, Wei-Ping Xiong, Jun-Li Zhang & Hui-Juan Sun X00486 (**Holotype**, BJTC FAN731; GenBank MF663714).

ETYMOLOGY—referring to the type locality.

ASCOMATA globose to subglobose, slightly lobed, firm, reddish brown when young, dark brown to blackish at maturity, 1.5–10 cm diam., with distinct pentagonal and pyramidal warts on the ascomatal surface, having 4–5 ridges ≤0.6 mm high. Odor aromatic at maturity. PERIDIUM 250–550 µm thick (excluding 300–600 µm high warts), two-layered: outer layer 225–300 µm thick, pseudoparenchymatous, composed of angular or irregular cells of (7.5–)12.5–30(–37.5) × (5–)7.5–15(–20) µm diam., thin-walled, walls gradually becoming brown towards outer surface; inner layer 200–300 µm thick, composed of hyaline interwoven hyphae, 2.5–5 µm diam., thin-walled, septate. GLEBA solid, whitish, light yellowish when young, gradually becoming brown to dark brown at maturity, marbled with numerous thin whitish meandering veins. ASCI globose, subglobose, or broadly ellipsoid, 62.5–75 × 50–62.5 µm, sessile at maturity, hyaline, thin-walled, 1–5-spored, randomly dispersed in glebal tissue. ASCOSPORES ellipsoid (usually a few broadly ellipsoid or subglobose), hyaline when young, yellow brown at maturity; excluding ornamentation 35–45 × 25–30 µm in 1-spored asci, 30–37.5 × 20–25 µm in 2-spored asci, and 20–32.5 × 17.5–22.5 µm in 3–5-spored asci, ornamented mostly by an irregular densely spiny reticulatum with occasional isolated spines, 2.5–4 µm tall, straight or strongly curved at apex.

ADDITIONAL SPECIMENS EXAMINED—**CHINA. TIBET, Linzhi City**, Bomi County, Yigong town, Jiangsegang village, 2230 m, in soil under *Pinus* sp., 26 July 2016, Wei-Ping Xiong, & Jun-Li Zhang X00454-1 (BJTC FAN728; GenBank MF663717), X00454-2 (BJTC FAN729; GenBank MF663716); 15 Nov. 2016, Wei-Ping Xiong, Jun-Li Zhang, & Hui-Juan Sun X00485 (BJTC FAN730; GenBank MF663715).

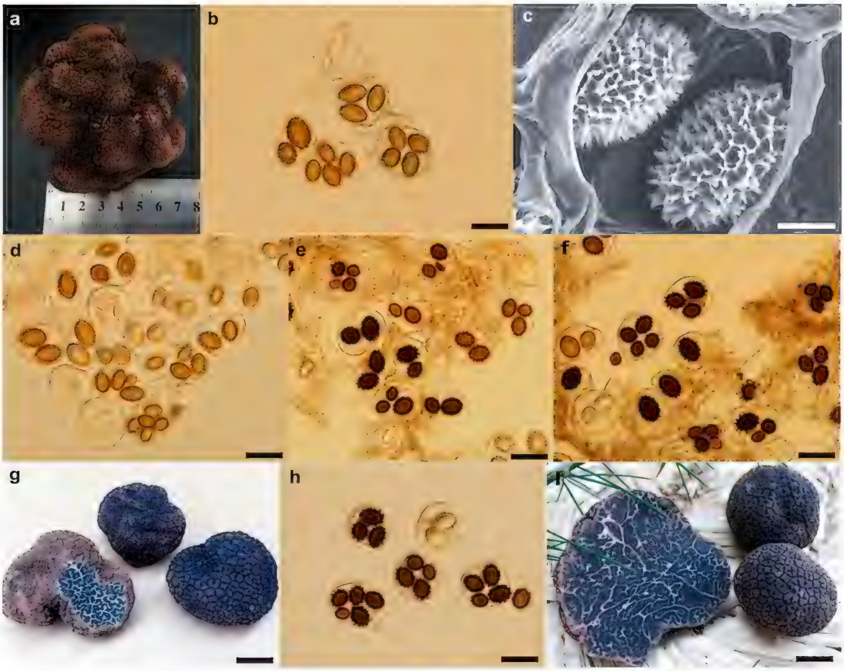


Fig. 2. Chinese *Tuber* species. ***Tuber yigongense*** (BJTC FAN731, holotype). a. Ascoma; b, d. Asci and ascospores (LM); c. Ascospores (SEM). ***Tuber formosanum*** (BJTC FAN720). e, f. Asci and ascospores (LM); g. Ascomata; ***Tuber sinense*** (BJTC FAN108). h. Asci and ascospores (LM); i. Ascomata. Scale Bars: a, g, i = 1 cm; b, d–f, h = 10 µm; c = 50 µm.

Comments—*Tuber yigongense* greatly resembles both *T. sinense* and *T. formosanum* in ascoma appearance as they all have large dark brown fruit-bodies covered by pyramidal warts. However, the yellow-brown ascospores of *T. yigongense* set it apart from *T. sinense* and *T. formosanum,* which have red-brown to blackish brown ascospores.

Although *T. yigongense* shares a <93% ITS sequence similarity with *T. sinense* and *T. formosanum* (Fig. 1), its sequences form a strongly supported (100% BS) monophyletic clade, supporting *T. yigongense* as a distinct species. Similarly, *Tuber indicum* and *T. himalayense* from India, *Tuber* sp. 7 from Japan, and *T. melanosporum* from Europe had similar ascomata to *T. yigongense* but differed by their black-brown or red-brown ascospores with spiny or sparsely spiny-reticulate ornamentations. Because there is no DNA data available from the two Indian *Tuber* species, their phylogenetic affinities with *T. yigongense* are yet to be resolved. We expect that additional collections *Tuber yigongense* will be found further afield, as more surveys are made in the area around Yigong.

Tuber sinense K. Tao & B. Liu,
J. Shanxi Univ. (Nat. Sci. Ed.) 12(2): 215 (1989) FIG. 2h,i
?MISAPPLIED NAME: *Tuber indicum* sensu auct. sin.

Tuber sinense, corresponding to Cluster A in ML tree (FIG. 1), was proposed by Tao & Liu (Tao & al. 1989) based on specimens from Huidong County, Sichuan Province, China. While this species is morphologically very similar to *T. indicum*, there is no molecular evidence that can confirm *T. indicum* and *T. sinense* are conspecific; all DNA data under the name of *T. indicum* in public databases and academic papers are derived not from Indian collections, but from China. We believe that "*T. indicum*" has been misapplied to Chinese specimens with Cluster A sequences, and that *T. sinense* is the appropriate name for Chinese specimens collected within Southwestern China.

Tuber formosanum H.D. Hu & Y. Wang, Mycotaxon 123: 296 (2013) FIG. 2,e–g
?MISAPPLIED NAME: *Tuber indicum* sensu auct. sin.

Tuber formosanum, corresponding to Cluster B in our ML tree (FIG. 1), was initially proposed by Hu (1992) but not typified. Recently, Hu & Wang (Qiao & al. 2013) validated the name from Taiwan. *Tuber formosanum* is easily confused morphologically with *T. sinense.* Based on our observations, potential diagnostic distinctions between the two species include [1] the number of ascospores per ascus (1–6 in *T. sinense* vs. usually 1–4 in *T. formosanum*) and (2) ascospore ornamentation (mostly isolated spines in *T. sinense* vs. spines usually with many more basal connections in *T. formosanum*). Although *T. formosanum* was previously treated as a synonym of *T. indicum* (Chen 2007, Cao 2010), our phylogenetic analysis supports *T. formosanum* as a good species.

According to our study, *T. formosanum* has been recorded from Taiwan, Sichuan, Yunnan, Jiangsu, and Hebei provinces in China, and from Japan (as *Tuber* sp. 6; Kinoshita & al. 2011) (TABLE 1, FIG. 1).

Discussion

Species diversity and geography of Eurasian black truffles

Our ITS-based phylogenetic analysis (FIG. 1) of Asian and European black truffles supports the existence of at least 5 distinct phylogenetic species: *Tuber melanosporum*, *T. formosanum*, *T. sinense*, *T. yigongense*, and *Tuber* sp. 7 (Japan). These species share many morphological features—large dark brown to blackish warty ascomata, a black gleba marbled with whitish vines, blackish or yellow brown (*T. yigongense*) ascospores ornamented with spines or a spine covered-reticulum—by which they are clearly separated from all other species in the genus. However, they are rather difficult to distinguish from one another using only morphological characters. One reason is that some important morphological features overlap among the species. For example, isolated spine ornamentation of spores is typical for *T. melanosporum*, but isolated spines (particularly on immature spores) are also often observed in *T. sinense;* similarly, spino-reticulate ascospore ornamentation, a diagnostic feature in *T. himalayense* (Zhang & Minter 1988), is also common and typical in both *T. yigongense* and *T. formosanum*. Secondly, some morphological characters often vary greatly within a species. For example, isolated ascospore spines predominate in some *T. sinense* specimens while the ornamentation is more spino-reticulate in others. Consequently, we think it is unworkable to identify black truffle species accurately without molecular data. *Tuber yigongense* might be an exception due to its generally pale yellow-brown ascospores, which make it easily distinguishable from other black truffle species.

Our molecular analysis suggests that *T. sinense* is limited to Southwestern China. *Tuber yigongense* has been found only in Tibet, and perhaps occurs in a region geographically isolated from the two other Chinese black truffles. In contrast, *T. formosanum* is widespread in Southwestern China (Yunnan and Sichuan provinces, where it overlaps with *T. sinense*), but it has also been collected from Taiwan (its type locality), Central China (Jiangsu province), Northern China (Hebei province), and Japan (FIG. 1). More recently, *T. formosanum* (as *Tuber indicum*-B, Bonito & al. 2011) was found fruiting in natural forest of North America, although its presence there might have been a deliberate introduction by human activity. However, a somewhat similar situation is observed in isolated pockets of *T. melanosporum* in southern Europe (Riousset & al. 2001, Hall & al. 2007) and *Tuber* sp. 7 (Japan) that appears native to Japan (Kinoshita & al. 2011). Thus, it seems probable that each known black truffle species is confined to a separate, relatively small area in the northern hemisphere.

Tuber indicum, T. himalayense, and Chinese black truffles

We exclude *T. indicum* and *Tuber himalayense* from the above discussion because the types (K39493, HMAS 68228) did not yield any useable DNA.

Their type locality is Mussoorie, near Dehradun in northern India on the southwestern Himalayan Plateau, which is separated from the type locality of *T. yigongense* by1600 km from Yigong on the northeastern part and the highest part of the Himalayan Plateau. This barrier and the distance between them and the other Asiatic species lead us to believe that *T. indicum* and *T. himalayense* might well be molecularly distinct from *T. yigongense* and the other species to the east.

Without supporting sequence data, it is unwise to assume that the Indian *T. indicum* and *T. himalayense* are conspecific with any of the Chinese black truffle species. For the time being, we think it is better to limit application of the names *T. indicum* and *T. himalayense* to Indian specimens, until authentic DNA material becomes available to resolve the taxonomic misunderstanding of the Chinese black truffles.

Acknowledgments

We are grateful to Prof. Tai-hui Li and Dr. Ian Hall for reviewing the manuscript. We express our great thanks to Dr. Jin-zhong Cao for his valuable comments on an earlier version of this paper. The study was supported by the National Natural Science Foundation of China (No. 31270058) and the Beijing Natural Science Foundation (No. 5172003).

Literature cited

Belfiori B, Riccioni C, Paolocci F, Rubini A. 2013. Mating type locus of Chinese black truffles reveals heterothallism and the presence of cryptic species within the *T. indicum* species complex. PLoS ONE 8: e82353. https://doi.org/10.1371/journal.pone.0082353

Bonito GM, Gryganskyi AP, Trappe JM, Vilgalys R. 2010. A global meta-analysis of *Tuber* ITS rDNA sequences: species diversity, host associations and long-distance dispersal. Molecular Ecology 19: 4994–5008. https://doi.org/10.1111/j.1365-294X.2010.04855.x

Bonito G, Trappe JM, Donovan S, Vilgalys R. 2011. The Asian black truffle *Tuber indicum* can form ectomycorrhizas with North American host plants and complete its life cycle in non-native soils. Fungal Ecology 4: 83–93. https://doi.org/10.1016/j.funeco.2010.08.003

Bonito G, Smith ME, Nowak M, Healy RA, Guevara G, Cázares E, Kinoshita A, Nouhra ER, Domínguez LS, Tedersoo L, Murat C, Wang Y, Moreno BA, Pfister DH, Nara K, Zambonelli A, Trappe JM, Vilgalys R. 2013. Historical biogeography and diversification of truffles in the *Tuberaceae* and their newly identified southern hemisphere sister lineage. PLoS ONE 8: e52765. https://doi.org/10.1371/journal.pone.0052765

Cao JZ. 2010. The genus *Tuber* in China. [Dissertation]. Changchun: Jinlin Agricultural University. 86 p. (in Chinese)

Castresana J. 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Molecular Biology and Evolution 17: 540–552. https://doi.org/10.1093/oxfordjournals.molbev.a026334

Chen J. 2007. Taxonomy and phylogeny of the genus *Tuber* in China (*Pezizales, Ascomycetes, Ascomycotina*). [Dissertation]. Beijing: Graduate School of Chinese Academy of Sciences. 125 p. (in Chinese)

Chen J, Guo SX, Liu PG. 2011. Species recognition and cryptic species in the *Tuber indicum* complex. PLoS ONE 6: e14625. https://doi.org/10.1371/journal.pone.0014625

Chen J, Murat C, Oviatt P, Wang Y, Tacon FL. 2016. The black truffles *Tuber melanosporum* and *Tuber indicum*. 19–32, in: A Zambonelli & al. (eds): True truffle (*Tuber* spp.) in the world: soil ecology, systematics and biochemistry. Berlin: Springer International Publishing. https://doi.org/10.1007/978-3-319-31436-5_2

Comandini O, Pacioni G. 1997. Mycorrhizae of Asian black truffles, *Tuber himalayense* and *T. indicum*. Mycotaxon 63: 77–86.

Cooke MC, Massee G. 1892. Himalayan truffles. Grevillea 20: 67.

Fan L, Han L, Zhang PR, Yan XY. 2016a. Molecular analysis of Chinese truffles resembling *Tuber californicum* in morphology reveals a rich pattern of species diversity with emphasis on four new species. Mycologia 108: 344–353. https://doi.org/10.3852/14-343

Fan L, Zhang PR, Yan XY, Li Y. 2016b. Phylogenetic analyses of Chinese *Tuber* species that resemble *T. borchii* reveal the existence of the new species *T. hubeiense* and *T. wumengense*. Mycologia 108: 354–362. https://doi.org/10.3852/14-349

Feng B, Zhao Q, Xu J, Qin J, Yang ZL. 2016. Drainage isolation and climate change-driven population expansion shape the genetic structures of *Tuber indicum* complex in the Hengduan Mountains region. Scientific Reports 6: 21811. https://doi.org/10.1038/srep21811

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. Molecular Ecology 2: 113–118. https://doi.org/10.1111/j.1365-294X.1993.tb00005.x

Hall IR, Brown GT, Zambonelli A. 2007. Taming the truffle. Portland: Timber Press.

Hillis DM, Bull JJ. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Systematic Biology 42: 182–192. https://doi.org/10.1093/sysbio/42.2.182

Hu HT. 1992. *Tuber formosanum* sp. nov. and its mycorrhizal associations. Quarterly Journal of Experimental Forestry, National Taiwan University 6: 79–86. (in Chinese)

Kinoshita A, Sasaki H, Nara K. 2011. Phylogeny and diversity of Japanese truffles (*Tuber* spp.) inferred from sequences of four nuclear loci. Mycologia 103: 779–794. https://doi.org/10.3852/10-138

Li Y, Li TH, Yang ZL, Bau T. 2015. Atlas of Chinese macrofungal resources. Zhengzhou: Central China Farmers Publication House.

Löytynoya A, Goldman N. 2005. An algorithm for progressive multiple alignment of sequences with insertions. PNAS 102: 10557–10562. https://doi.org/10.1073/pnas.0409137102

Page RDM. 2001. TreeView 32 1.6.6. Distributed by the author [available on internet at http://taxonomy.zoology.gla.ac.uk/rod/rod.html].

Qiao P, Liu PG, Hu HT, Wang Y. 2013. Typification of *Tuber formosanum* (*Tuberaceae, Pezizales, Ascomycota*) from Taiwan, China. Mycotaxon 123: 293–299. https://doi.org/10.5248/123.293

Riousset L, Riousset G, Chevalier G, Bardet MC. 2001. Truffes d'Europe et de Chine. Paris: Institut National de la Recherché Agronomique.

Song MS. 2005. Taxonomic and molecular systematic studies on *Tuber* in China. [Dissertation]. Beijing: Graduate School of Chinese Academy of Sciences. 156 p. (in Chinese)

Stamatakis A. 2006. RAxML-VI-HPC: maximum-likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22: 2688–2690. https://doi.org/10.1093/bioinformatics/btl446

Stamatakis A. 2014. RAxML Version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30.9(2014): 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Stamatakis A, Ludwig T, Meier H. 2005. RAxML-III: a fast program for maximum likelihood-based inference of large phylogenetic trees. Bioinformatics 21: 456–463. https://doi.org/10.1093/bioinformatics/bti191

Tao K, Liu B, Zhang DC. 1989. A new species of the genus *Tuber* from China. Journal of Shanxi University (Natural Science Edition) 12: 215–218.

Wang YJ, Tan ZM, Zhang DC, Murat C, Jeandroz S, Tacon FL. 2006. Phylogenetic and populational study of the *Tuber indicum* complex. Mycological Research 110: 1034–1045. https://doi.org/10.1016/j.mycres.2006.06.013

White TJ, Bruns TD, Lee SB, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. 315–322, in: MA Innis & al. (eds). PCR protocols: a guide to methods and applications. San Diego, CA. Academic Press. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Zambonelli A, Tibiletti E, Pisi A. 1997. Caratterizzazione anatomo-morfologica delle micorrize di *Tuber indicum* Cooke & Massee su *Pinus pinea* L. e *Quercus cerris* L. Micologia Italiana 26: 29–36.

Zang M, Pu CX, Wu JM, Chen QF, Liu B. 1992. A confirmatory *Tuber indica* distributed in China. Edible Fungi of China 11(3): 19, 39. (in Chinese)

Zhang BC, Minter DW. 1988. *Tuber himalayense* sp. nov. with notes on Himalayan truffles. Transactions of the British Mycological Society 91: 593–597. https://doi.org/10.1016/S0007-1536(88)80032-9

Zhang LF, Yang ZL, Song DS. 2005. A phylogenetic study of commercial Chinese truffles and their allies: taxonomic implications. FEMS Microbiological Letters 245: 85–92. https://doi.org/10.1016/j.femsle.2005.02.028

Zhuang WY. 1998. A list of discomycetes in China. Mycotaxon 67: 365–390.